REMARKS

Claims 1-6 and 10-12 are currently pending and stand rejected.

Claims 1 and 5 have been amended. Support for the amendment to claim 1 can be found at specification at page 5, lines 31-34, page 15, lines 13-17 and page 16, lines 16-24. Support for the amendment to claim 5 can be found in the specification at page 18, lines 29-34. No new matter has been added by the amendments.

The Office Action withdrew all previous rejections under 35 U.S.C. §112, first paragraph, 35 U.S.C. §112, second paragraph and 35 U.S.C. §102 (see Office Action, page 2, paragraph 3).

Please reconsider the Application in light of the following remarks.

I. Objection to Claims 5, 6 and 10

Claim 5 and dependent claims 6 and 10 were objected to. The Office Action requested Applicants to amend claim 5 to recite "the composition further comprises GM-CSF" in order to clarify that the composition also includes GM-CSF (Office Action, page 2, paragraph 4). Applicants have amended claim 5 as requested. Accordingly, the objection to claims 5, 6 and 10 may be properly withdrawn.

II. The Claims Meet the Requirements of 35 U.S.C. § 112, First Paragraph

Claim 1 and dependent claims 2-6 and 10-12 stand rejected under 35 U.S.C. § 112, first paragraph, for an alleged insufficient written description to show that Applicants were in possession of the claimed invention. Specifically, the Office Action states that the application does not provide support for the invention, wherein the maturation factor is present in macrophage conditioned medium (Office Action, pages 2-3, part 6).

In order to expedite prosecution, Applicants have amended claim 1 to delete the term "macrophage conditioned medium". Applicants reserve the right to prosecute claims directed to this subject matter in a continuation application. Accordingly, Applicants respectfully submit that this rejection of claims 1-6 and 10-12 under 35 U.S.C. §112, first paragraph, may be properly withdrawn.

Claim 1 and dependent claims 2-6 and 10-12 stand rejected under 35 U.S.C. § 112, first paragraph, as allegedly not enable for a method comprising contacting the pluripotential cells having the potential of expressing either macrophage or dendritic cell characteristics with one or more cytokines for a time sufficient to produce immature dendritic cells . . .". The Office Action acknowledges that the claims are enabled where the cytokines are GM-CSF and IL-4, but alleges that "no other cytokines or combinations thereof are even disclosed" (Office Action, page 3-4, part 7).

Applicants have amended claim 1 to specify that the pluripotential cells are contacted with "the combination of GM-CSF and IL-4 or IL-13. Applicants respectfully disagree that no cytokines or combinations thereof other than GM-CSF and IL-4 are disclosed. The specification discloses the combination of GM-CSF with IL-4 *or IL-13* for the production of immature dendritic cells from pluripotential cells having the potential of expressing either macrophage or dendritic cell characteristics (see specification at page 5, lines 31-34, page 15, lines 13-17 and page 16, lines 16-24). Although use of the combination of GM-CSF and IL-13 is not reported in the examples, it was known at the time of filing that IL-13 could substitute for IL-4 in the differentiation of dendritic cells from pluripotential cells having the potential of expressing either macrophage or dendritic cell characteristics. For example, Piemonti et al. (1995) Eur Cytokine Netw 6:245-252 (attached as Appendix A) concludes that "IL-13 is as effective as IL-4, combined with GM-CSF is sustaining DC differentiation from PBMC . . ." (Abstract, page 245). Furthermore, Cohen et al. (1996), which was cited at page 4 of the Office Action, discloses at page 591, column 2, that "IL-4 shares most biologic activities on B cells, monocytes, NK cells, and endothelial cells with Interleukin-13 (IL-13)." Accordingly, the instant application, at the time of filing, clearly contained sufficient information regarding the combination of GM-CSF with IL-4 or IL-13 for the production of immature dendritic cells from pluripotential cells having the potential of expressing either macrophage or dendritic cell characteristics, to enable one of skill in the art to practice the invention as claimed in amended claim 1 without undue experimentation. Thus, the rejection of claims 1-6 and 10-12 stand under 35 U.S.C. § 112, first paragraph, for lack of enablement, may be properly withdrawn.

CONCLUSION

Applicants respectfully submit that the claims are in condition for allowance. However,

if the Examiner believes that any further discussion of this communication would be helpful, he is encouraged to contact the undersigned by telephone.

No fees are believed to be due in connection with this communication. Should any addition charges be due, please apply them to our Deposit Account No. 50-3187.

Respectfully submitted,

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APPENDIX A

Eur. Cytokine Netw., Vol. 6 n°4, *July-December 1995*, 245-252

IL-13 supports differentiation of dendritic cells from circulating precursors in concert with GM-CSF

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ABSTRACT. Dendritic cells (DC) can be obtained from peripheral blood mononuclear cells (PBMC) by in vitro culture with IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF). IL-13 shares properties with IL-4 but its receptor does not involve the common γ chain present in the receptor complex of IL-4 and other cytokines. The present study was aimed to elucidate whether IL-13 can substitute for IL-4 in DC cultures and to compare the phenotypic and functional characteristics of cells obtained using these two cytokines. Monocyte-enriched PBMC were cultured with GM-CSF and IL-4 or GM-CSF and IL-13. Cell yields and DClike morphology were similar. The cells showed a membrane phenotype typical of DC (MHC II$^+$; CD1a$^+$; CD14$^-$; CD3$^-$; CD20$^-$). IL-13-derived and IL-4-derived DC were similar in terms of macropinocytosis, stimulatory capacity of cord blood lymphocytes in mixed leukocyte reaction (MLR), and responsiveness to chemotactic signals. It is concluded that IL-13 is as effective as IL-4, combined with GM-CSF in sustaining DC differentiation from PBMC and that activation of the common γ chain-driven transduction pathways is dispensable for DC differentiation and function.

Keywords: Monocytes, Dendritic Cell, Cytokine, IL-13, IL-4, GM-CSF

INTRODUCTION

Dendritic cells represent an heterogeneous population of bone-marrow-derived cells specialized in Ag presentation to naive [1] and resting T lymphocytes [2]. Being the most potent Ag presenting cell (APC) *in vitro* and *in vivo*, they play a key role in the initiation of the immune response and are considered promising tools and targets for immunotherapy [3 - 4]. DC are scattered all over the body in lymphoid as well as non lymphoid organs [5]. Until recently DC were isolated from tissues [6 -11] or blood [12 - 14] with enrichment protocols which yielded relatively few cells [15]. In the last years a number of new procedures have been developed for obtaining DC including generation from human peripheral blood [16] and bone marrow [17] CD34$^+$ hematopoietic progenitor cells in the presence of tumor necrosis factor alpha (TNF-α) and GM-CSF; generation from PBMC by culturing in particular microenvironmental: serum-free conditions [18]; serum-free and IL-6 [19]; serum-free and IL-4 [20], GM-CSF and IL-4 [21]. DC obtained with GM-CSF and IL-4 show functional and phenotipic characteristics of immature dendritic cells (i.e. high capacity of Ag uptake and processing) and can be further differentiated *in vitro* into mature DC with TNF-α and LPS [21].

The role played by IL-4 in the development of DC from adult PBMC is not clear [18]. Human IL-13 is a secreted protein of 112 amino acids showing approximately 30% amino acid sequence homology with IL-4 [22] and is encoded by a member of the GM-CSF, IL-5, and IL-4 gene cluster [23]. IL-4 and IL-13 map on chromosome 5q 23-31 and show homology in the first and last α-elicae regions which are critical for their activity [22]. IL-13 shows a spectrum of action which overlaps with but yet is clearly distinct from that of IL-4. Similarly to IL-4, it induces IgG4 and IgE synthesis and expression of CD23 by human B cells [24], inhibits monokine release [22], upregulates CD23 and MHC II on human monocytes [24 - 25], induces expression and release of interleukin-1 decoy receptor in human polymorphonuclear cells [26], induces VCAM-1 and amplifies IL-6 production in endothelial cells and mesothelial cells [27], inhibits the generation of procoagulant activity in monocytes and endothelial cells [28]. However, IL-13 differs from IL-4 in several properties. First, at variance with the action of IL-4 [29 - 30], human IL-13 does not inhibit the production of interferon-γ (IFN-γ) by NK cells *in vitro* [22]; on the contrary, it synergizes with IL-2 to stimulate IFN-γ production by NK cells [22]. Second, in contrast to IL-4, IL-13 fails to activate human T cells [31], has no

growth-promoting effects on PHAactivated T-cell blasts or activated CD4+ or CD8+ T-cell clones, and is not effective in inducing CD8α expression on CD4+ T-cell clones or CD4+ T cells isolated from cord blood [31]. Finally no detectable binding of radiolebeled IL-13 has been found on activated T cells [31].

The IL-13 and IL-4 receptors share components of the receptor complex [32]. However, the γ chain, common to IL-4, IL-2, IL-7, IL-9 and IL-15 receptors and functionally coupled to Jak-3 kinase, is not part of the IL-13 receptor complex [33 - 37]. It was therefore of interest to investigate wheter IL-13 shares the capacity of IL-4 to drive DC differentiation. In this study we demonstrate that low density mononuclear cells enriched in monocytes cultured with GM-CSF and IL-13 differentiate into DC with similar typical dendritic morphology, phenotype and functional activity as compared to those obtained with GM-CSF and IL-4.

MATERIALS AND METHODS

Cytokines. Human recombinant GM-CSF was from Sandoz (Basel, Switzerland). IL-4 was kindly provided by Immunex (Seattle, WA). Interleukin 13 (IL-13) and MCP-3 were from Sanofi Elf Bio Recherches, Labège, France. Human recombinant MCP- 1 was from Peprotech Inc . (Rocky Hill, NJ, USA). Human recombinant MIP-1a/LD 78 was a kind gift from Dr. L. Czaplewsky, British Bio-technology Limited, Cowley, UK. Nformyl methionyl leucyl phenylalanine (fMLP) was from Sigma (St. Louis, MO). Human recombinant TNFa was from BASF (Knoll, Germany).

Preparation of DC. Low density peripheral blood PBMC highly enriched in monocytes were obtained and purified from buffy coats (through the courtesy of Centro Trasfusionale, Ospedale Sacco, Milan, Italy) by Ficoll and Percoll gradients. Cells were cultured for 7 days at 5 x 10^5/ml in 6-well tissue culture plates (Falcon, Becton Dickinson, New Jersey) in RPMI (Biochrom, Berlin, FRG) 10% FCS (Hyclone, Logan, UT) supplemented with 50 ng/ml GM-CSF and 50 ng/ml IL-4 or IL-13. After 7 days cells were harvested, washed and counted. Usually 50-70% of the starting population was recovered. Cells were further depleted of monocytes, B and T lymphocytes by CD14, CD19, and CD2 coated Dynabeads (Unypath, Milan, Italy) before use in experiments.

FACS Analysis. Cell staining was performed using mouse monoclonal antibodies followed by FITC-conjugated affinity purified goat anti-mouse antibodies (Techno-Genetics). The following mAbs were used: L243 (IgG2a, anti DR); OKT3 (IgG2a, anti CD3); TS 1/18 (IgG2a, anti CD18); TS1/22 (IgG2a, anti CD11a); B1 (IgG2a, anti CD20) were all purchased from ATCC (Rockville, MD); NA1/34 (IgG2a, anti CD1a) from Dako A/S, Denmark. The following mAbs were obtained through . the courtesy of collegues: UCH M1 (IgG2a, anti CD14) from Dr. P. Beverly, London UK; CL 207 (IgG2a, anti ICAM1) from Dr. S. Ferrone, New York, NY; MO1 (IgG2a, anti CD11b), from Dr. R. Todd, Ann Arbor, MI; L29 (IgG1, anti CD11c) from Dr. L. Lanier, DNAX Palo Alto, CA. Control cells were incubated with a primary irrilevant antibody (IFGCP, anti IFN-γ IgG1) Results are expressed as % of positive cells and as relative fluorescence intensity (RFI), calculated according to the formula:

$$RFI = \frac{\text{mean fluorescence (sample)-mean fluorescence (control)}}{\text{mean fluorescence (control)}}$$

Mixed leukocyte reaction (MLR). Freshly isolated monocytes and DC cultured with GM-CSF+IL-4 or GM-CSF+IL-13 obtained from the same donor were irradiated (3,000 rad) and added in graded doses to 1 x 10^5 purified allogenic T cell in 96-well flat-bottomed microtest plates. T cells were depleted of autologous APC by passage with CD14 and CD19 coated Dynabeads (Unypath, Milan, Italy). Each group was performed in triplicate. 3H-thymidine incorporation was measured on day 5 by 16 h pulse (5 ci/llmole, Amersham, UK).

Pinocytosis. Pinocytosis was measured as the cellular uptake of FITC-dextran (38) and quantitated by flow cytometry. Aproximately 2 x 10^5 cells sample were incubated in medium containing dextran or FITCdextran (molecular weight 70,000 Sigma). We previously determined the appropriate conditions for a flow cytometric assay of pinocytosis. The FITC-dextran concentration of 1 mg/ml was selected from the midpoint of the linear portion of the curve from a dose-response experiment (data not show), whereas the optimum incubation time (60 and 120 min) was determined from a time-course experiment (data not show). After incubation cells were washed twice with PBS to remove excess dextran and fixed in cold 1% formalin. The quantitative uptake of FITC-dextran by the cells was determined using a FACS [39]. At least 8,000 cells per sample were analyzed. Each group was performed in triplicate. In some experiments cells were preincubated for 20h with TNFa (20 ng/ml).

Migration assay. Cell migration was evaluated using a chemotaxis microchamber technique. Twenty-five microliters of chemoattractant solution or control medium (RPMI 1641 with 1% FCS) were added to the lower wells of a chemotaxis chamber (Neuroprobe, Pleasanton, CA). A polycarbonate filter (5 µm pore size; Neuroprobe) was layered onto the wells, covered with a silicon gasket and with the top plate. Fifty microliters of cell suspension (0.7-1 x 10^6/ml) were seeded in the upper chamber. The chamber was incubated at 37°C in humidified atmosphere in the presence of 5% $C0_2$ for 90° min. At the end of the incubation the filter was removed, stained with Diff-Quik (Baxter s.p.a., Rome, Italy) and five high power oil immersion fields (100 x) were counted. Results are expressed as the mean number of migrated cells or as percentage of input cells migrated. Each group was performed in triplicate.

RESULTS

Morphology and phenotype. Low density mononuclear cells highly enriched in monocytes (70-90% CD 14+cells) were cultured with GM-CSF and either IL-4 or IL-13. After 7 days cell recovery for both cytokine combinations was usually 50-70% of the cells originally plated. DC were purified by passage with magnetic beads coated with CD14, CD2 and CD19 antibody.

By light microscopy, DC obtained with GM-CSF and IL-4 or GM-CSF and IL-13 were of large size and showed the same typical dendritic morphology (Fig. 1). Cells were loosely or non adherent to plastic and showed long cytoplasmatic processes.

A representative experiment of surface phenotype of DC cultured with GM-CSF+IL-4 or GM-CSF+IL-13 from the same donor is shown in Fig. 2. In both conditions DC expressed high levels of CDla and of HLA-DR, and lacked markers typical of monocytes, B and T cells (CD14, CD20 and CD3). Analysis of surface adhesion molecules showed that both types of

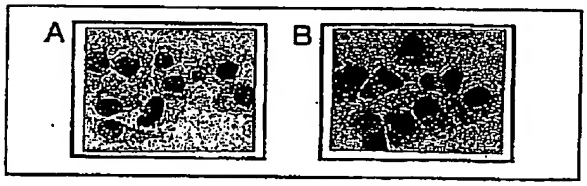

Figure 1
Morphology of DC obtained by culturing with IL-13
DC were cultured for 8 days with (A) GM-CSF and IL-4 or (B) GM-CSF and IL-13 . In both cultures cell were of large size and showed cytoplasmic protrusion typical of DC. Cyto-preps were stained with May-Grunwald-Giemsa. Magnification 400x.

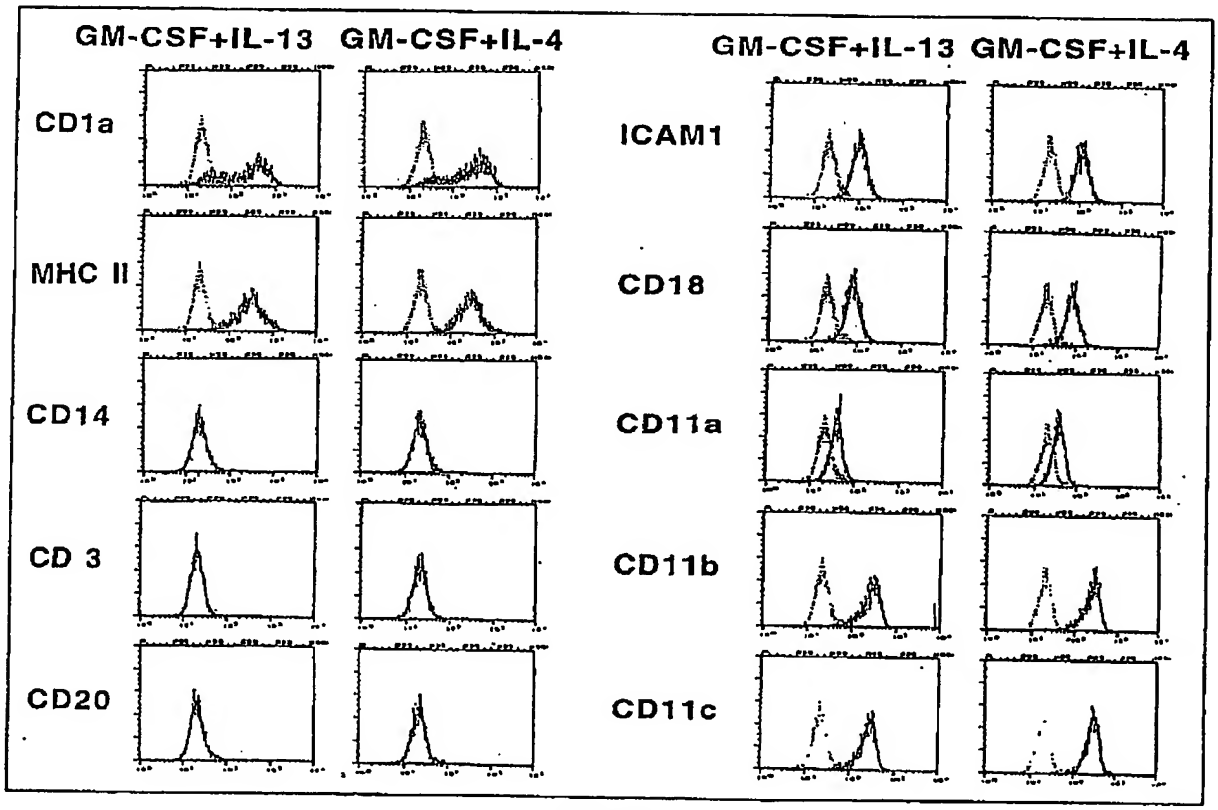

Figure 2
Surface phenotype of DC obtained from PBMC with IL-13.
Cells from the same donor were cultured with GM-CSF+IL-4 or GMCSF+IL-13. On day 8 cells were depleted of monocytes, B and T cells by CD14, CD19 and CD2 coated Dynabeads. Cell staining was performed using mouse monoclonal antibodies followed by FITCconjugated goat anti-mouse antibodies.

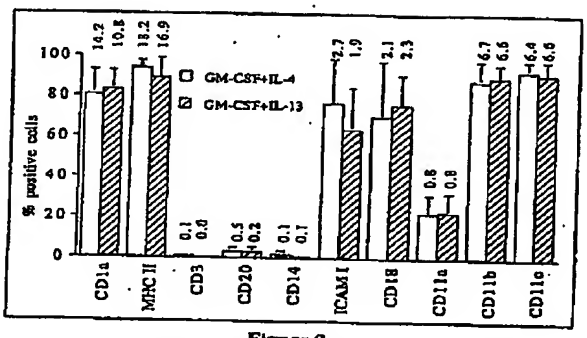

Figure 3
Summary of surface marker of IL-13 cultured DC. Analysis of surface molecules from all the experiments performed with GM-CSF+IL-13 (n = 6) and GM-CSF+IL-4 (n = 9) . The results are expressed as mean % positive cells ± SE. Numbers on top of bars indicate values of relative fluorescence intensity (RFI).

DC expressed high levels of CD11b and CD11c, intermediate levels of CD18 and ICAM1, low levels of CD11a. Fig. 3 summarizes the data of 6 experiments with GM-CSF+IL-13 and 9 experiment with GM-CSF+IL-4. Thus IL-13 in concert with GM-CSF is as effective as IL-4 in inducing differentiation from PBMC of cell with dendritic morphology and phenotype.

Stimulatory capacity of DC in MLR. DC cultured with GM-CSF+IL-4 or GM-CSF + IL13 and freshly isolated monocytes from the same donor were compared for their capacity to stimulate purified allogeneic T cells. DC cultured in both conditions were strong stimulator of T cells proliferation (Fig. 4). At the stimulator/responder (S/R) ratio of 1: 128 DC cultured with GM-CSF+IL-4 and those cultured with GM-CSF+IL-13 had a proliferative index of 32 and 26, respectively, compared to. an index of 5 for monocytes. It is well established that only DC but not other APC are able to induce proliferation of cord blood lymphocytes in MLR. It has been already reported that DCs cultured with GM-CSF+IL-4 induce cord blood T cell proliferation. Fig. 5 shows that DC obtained with GM-CSF+IL-13 are good stimulators of cord blood lymphocytes, while monocytes from tha same donor were inactive: at the S/R ratio of 1/4 DC cultured with GM-CSF+IL-13 and monocytes had a proliferative index of 64 and 6, respectively.

Pinocytosis. DC cultured in GM-CSF+IL-13 showed a strong pinocytotic activity that was about 10-fold greater than that of monocytes and 50 - 60-fold than that of lymphocytes, a repressentative experiment is shown in Fig. 6, panel A. In another experiment we compared the pinocytic activity of DC obtained with GM-CSF+IL-4 and GM-CSF+IL-13. Uptake of FITC -dextran was similar for the two DC preparations

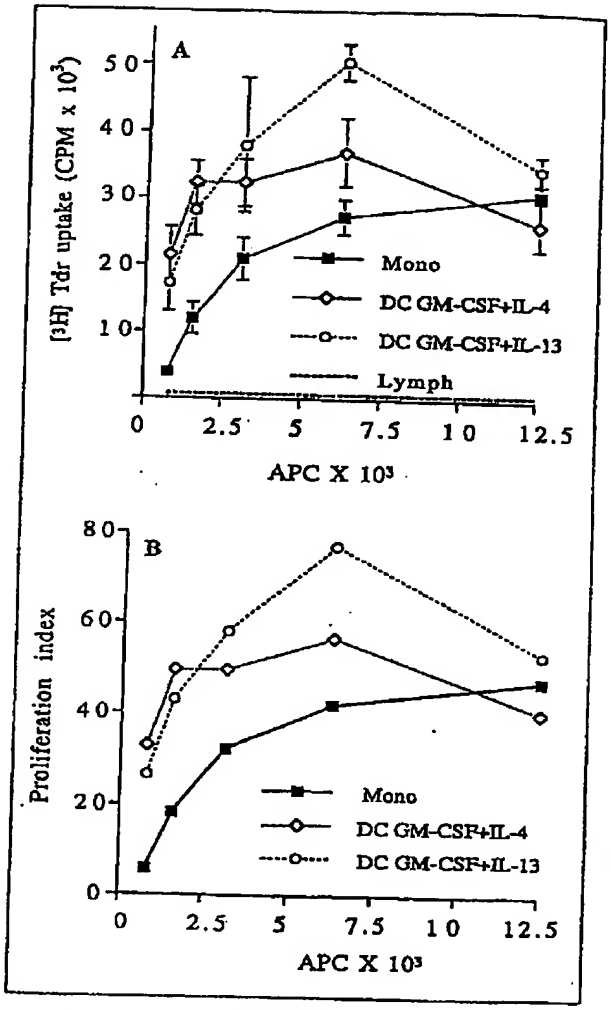

Figure 4
Stimulatory activity of IL-13 cultured DC in MLR. Adult T cells cultured with different numbers of allogeneic monocytes and DCs from the same donor obtained with GM-CSF+IL-4 or GM-CSF+IL-13. The proliferative response was measured on day 5 by 3H Thymidine uptake (panel A) or proliferation index (panel B).

during the 2h exposure (Fig. 6B). As previously reported [40], when preincubated for 20h at 37° with 10 ng/ml TNF-α the uptake of FITC-dextran was inhibited of about 50% for DC population. The inibitory activity of TNF-α was lost after boiling.

Migration assay. We previously reported that PBMC-derived DC cultured with GM-CSF+IL-4 migrate in chemotaxis assay in response to C5a, fMLP and a distinct set of C-C chemokines [41]. Fig. 7 shows that

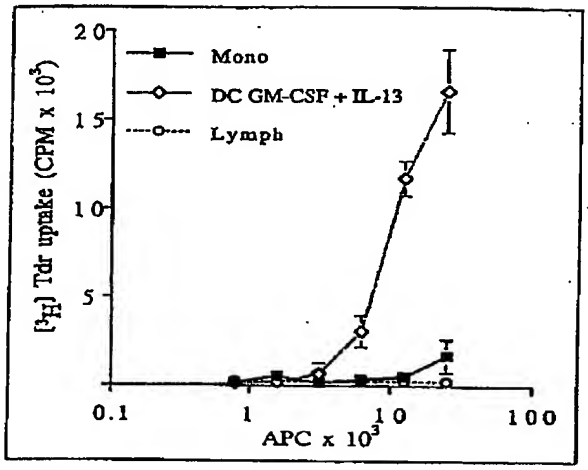

Figure 5
Stimulatory activity of IL-13 cultured DC with cord blood naive T cells.
Cord blood T cells were cultured with different number of allogeneic monocytes or DC from the same donor obtained with GM-CSF+IL-13 . The proliferative response was measured on day 5 by 3H Thymidine uptake.

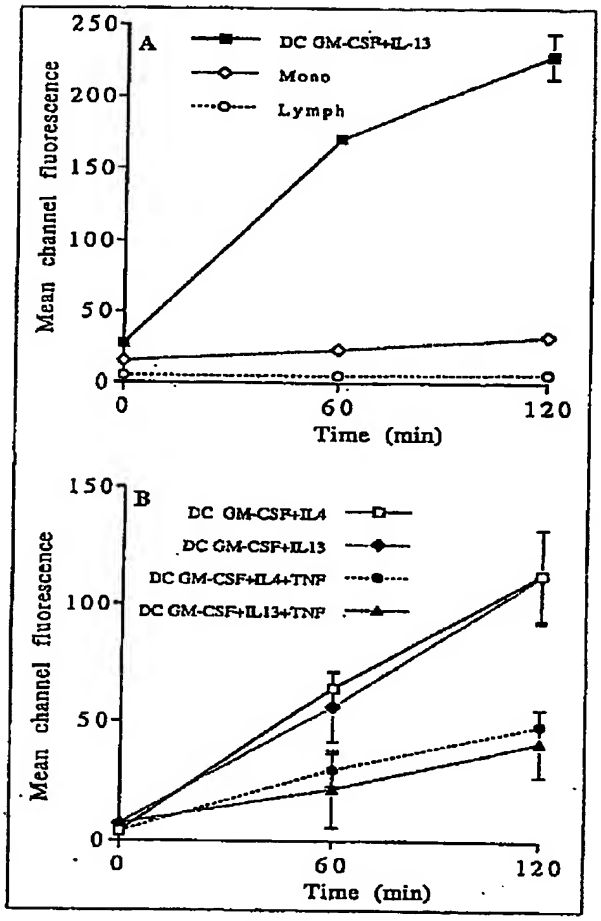

Figure 6
Endocytic activity of IL-13 cultured DC by uptake of FITC-Dextran.
Cells were incubated with 1 mg/ml FITC-Dextran for 60 or 120 min and analyzed on a FACScan. Results are expressed as mean channel fluorescence. Panel A and B refer to two different experiments. Panel A: pinocytic activity of cultured DC (GM-CSF+IL-13) compared to freshly isolated monocytes and lymphocytes from the same donor. Panel B: comparison of pinocytotic activity in DC obtained with GM-CSF+IL-4 or GM-CSF+IL-13. Cells were pre-treated with TNF-α (10 ng/ml) for 20 h

both type of DC migrate to fMLP and to MCP-3 and MIP-1a/LD78 but not to MCP-1. The pattern of chemotactic activation of DC cultured with GM-CSF+IL-13 in response to chemokines was similar to that obtained with GM-CSF+IL-4.

DISCUSSION

Isolation of a large number of DC with a high degree of purity is critical for the functional characterization of this cell type and for the identification of strategies for immune intervention. It was previously reported that culture of PBMC with GM-CSF and IL-4 allows the differentiation of DC in an immature state with high capacity of Ag uptake and processing [21]. In this study we have investigated whether GM-CSF combined with IL-13, a cytokine sharing a number of IL-4-like activities, allows the differentiation *in vitro* of human DC with the same characteristics . When IL-4 or IL-13 were associated with GM-CSF they had identical capacity to induce differentiation of PBMC to DC. Characterization of the cells was based on three diffrent characteristics well established and accepted for DC:

1) DC cultured in both conditions had the same typical morphology of large cells with long cytoplasmic protrusions, high expression of CD 1 a and MHC II, and low or absent expression of CD14;

2) both types of DC cultures were strong stimulators of proliferation for allogeneic adult T cells and, most importantly, triggered proliferation of cord blood T lymphocytes;

3) both types of DC had high endocytic activity evaluated as uptake of FITC-dextran and both types of DC responded to TNFa with changes that included a downregulation of pinocytosis concomitant with an upregulation of adhesion and costimulatory molecules (data not shown). These results demonstrate that IL-13 (combined with GM-CSF) can be used to generate DC from peripheral blood with identical results compared to IL-4 and GM-CSF.

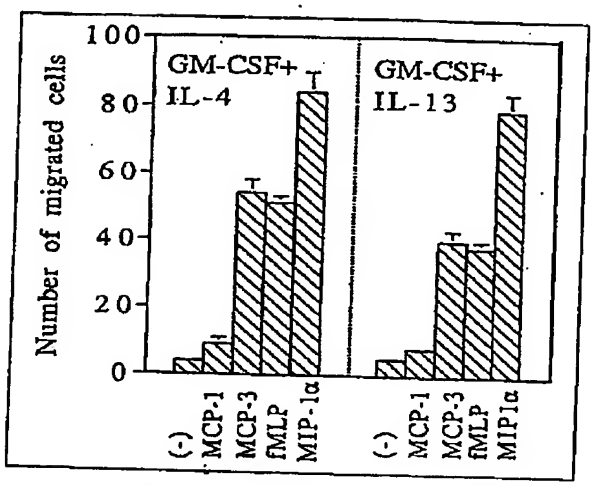

Figure7
Migratory activity of IL-13 cultured DC to chemokines and classical chemotactic factors through policarbonate filters. Chambers were incubated at 37°C for 90'. Results are expressed as the mean number (± SD) of migrating cells for five high power fields from one representative experiment.

We have also analyzed the *in vitro* ability of DC to migrate in response to chemotactic factors and to chemokines. In a previous study we have defined a panel of chemotactic signals able to induce directional migration of DC *in vitro* [41]. These included two prototypic leukocyte attractants, C5a and fMLP and members of the C-C chemokine family. The set of chemokines active on DC was different from that active on classical myelomonocytic cells; it included the C-C chemokines MCP-3, MIP-1a/LD78 and RANTES, but not MCP-1 and MCP-2. IL-8 and IP-10 (C-X-C family) were inactive. DC obtained with GM-CSF-IL-13 showed the same panel of response to chemotactic signals; it is particularly noteworthy that MCP-1, active on monocytes, is not active on DC.

It is not clear what role IL-4 and IL-13 may play, but one possibility is that they antagonize the effect of other cytokines acting as maturation-inducing signals. GM-CSF and LPS act as activation isgnals for TNF-α, while IL-4 suppresses TNF-α protein production and mRNA accumulation [42]. The inhibitory effect of IL-4 is mediated at least in part through the repression of promoter activity [42]. As TNF-α plays a role as one of maturation-inducing signals for DCs [43 - 44], it is tempting to speculate that IL-4 and perhaps also IL-13, could maintain DCs in an immature state by antagonizing TNF-α induction mediated by GM-CSF.

The actual *in vivo* significance of the capacity of IL-13 to drive DC differentiation remains unclear. IL-13 gene transfer has been shown to cause tumor regression and to generate specific anti-tumor immunity [45]. Along the same line Pardoll et al. have reported that transfer of GM-CSF gene (the other cytokine important for DC differentiation) in B16 murine melanoma confer resistance to tumor uptake and stimulate T cell mediated antitumor immunity [46]. The authors' speculation is that GMCSF triggers resident DC to present tumor-associated Ag to T cells, and similar mechanisms may be operative in IL-13-transfected tumors.

The common γ chain is part of the IL-4 receptor complex [34]. The IL13 receptor shares a component with that of IL-4 [32], but the common γ chain is not part of the IL-13 receptor [35 - 37, 47 - 48]. The finding that the non-chain cytokine IL-13 substitutes for IL-4 in sustaining DC generation from PBMC suggests that the common γ chain-driven transduction pathway is dispensable for DC differentiation under these conditions .

ACKNOWLEDGEMENTS. W. Trobonjaca is a visiting scientist from Dept. of Physiology and Immunology, Medical Faculty, University of Rijeka, Croatia. We thank Dr.Silvano Sozzani for helpful discussion This work was supported by Italian Association for Cancer Research and by National Research Council (CNR) strategic project Cytokines and finalized project ACRO

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